Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Inhibitory effect of St. John's Wort oil macerates on TNFα-induced NF-κB activation and their fatty acid composition

Ilkay Erdogan Orhan [a,*], Murat Kartal [b], Ali Rifat Gülpinar [b], Gülin Yetkin [a], Barbora Orlikova [c,d], Marc Diederich [c], Deniz Tasdemir [e,**]

[a] *Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 06330 Ankara, Turkey*
[b] *Department of Pharmacognosy, Faculty of Pharmacy, Ankara University, 06100 Ankara, Turkey*
[c] *Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*
[d] *Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, L-2540 Luxembourg, Luxembourg*
[e] *School of Chemistry, National University of Ireland Galway, Ireland*

A R T I C L E I N F O

Article history:
Received 9 April 2014
Received in revised form
11 June 2014
Accepted 12 June 2014
Available online 30 June 2014

Keywords:
Hypericum perforatum
St. John's Wort oil
Oleum Hyperici
Fatty acid
NF-κB activation
TNFα

A B S T R A C T

Ethnopharmacological relevance: The oil macerates of *Hypericum perforatum* L. (St. John's Wort=SJW) have a long history of medicinal use and SJW has been used in traditional medicine both orally and topically for centuries worldwide mainly for wound healing, ulcer and inflammation.
Materials and methods: We analyzed the fatty acid composition of 10 traditionally (home-made) and 13 commercially (ready-made) prepared SJW oil macerates by GC–MS. The acid, peroxide, iodine, saponification values, and the unsaponifiable matters of the samples were determined according to the European Pharmacopoeia. We also explored potential mechanism of wound healing effect of the samples, i.e. TNFα-induced NF-κB activation.
Results: The most home-made oil samples contained oleic acid predominantly and complied with the requirements set for olive oil by European Pharmacopoeia. However, majority of the ready-made samples appeared to have adulteration with some other oils. Moderate NF-κB inhibitory effects have been observed with some of the oil samples.
Conclusion: This study sheds light on the fact that application of the proper traditional method to prepare olive oil macerates from *Hypericum perforatum* is able to get bioactive constituents in the oil. Besides, inhibition of TNFα-induced NF-κB activation appears to be a potential mechanism for topical wound healing activity of SJW oil macerates.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Hypericum perforatum L. (Hypericaceae), also known as St. John's Wort (SJW) or 'binbirdelik otu' or 'sari kantaron' in Turkish, is one of the most intensively studied medicinal plants with a well-characterized phytochemistry. The best-known chemical constituents of the plant are the naphthodianthrone derivatives (hypericin and pseudohypericin), acylated phloroglucinol derivatives (hyperforin and adhyperforin), flavonoids (quercetin, quercitrin, hyperoside, rutin, kaempferol), and biflavonoids (biapigenin and amentoflavone). Standardized extracts, usually based on hypericin and pseudohypericin, of this plant are widely consumed for the treatment of mild to moderate type of depression (Francis, 2005). SJW has been used in traditional medicine both orally and topically for centuries worldwide, and is a very popular folk remedy in Anatolia. The decoction (rarely infusions too) of the plant is consumed against various ailments, e.g. uro-genital inflammations, diabetes, neuralgia, heart diseases, gastritis, hemorrhoids, and peptic ulcers (Yesilada et al., 1995). Another common usage form of the plant is the oil maceration, which is also described as the 'sunlight maceration method' (Maisenbacher and Kovar, 1992). This macerate (Oleum Hyperici) is prepared by soaking the fresh flowering tops of the plant into olive oil, which is rich in fatty acids, polyphenols such as hydroxytyrosol and its derivatives as well as tocopherols (Franco et al., 2014), and are abundant components in the olive oil, and are exposed it to sunlight for two to four weeks. The resulting dark red-colored infused oil named as 'sari kantaron yagi' is widely sold in traditional herbal stores in Turkey and used externally for its antiseptic and wound healing effects, particularly for rapid recovery of wounds, burns, and cuts (Baytop, 1984). Several studies have

* Corresponding author. Tel./fax: +90 312 2235018.
** Corresponding author. Tel.: +353 91 492450; fax: +353 91 495576.
E-mail addresses: iorhan@gazi.edu.tr, ilkay.orhan@emu.edu.tr (I.E. Orhan), deniz.tasdemir@nuigalway.ie (D. Tasdemir).

http://dx.doi.org/10.1016/j.jep.2014.06.030
0378-8741/© 2014 Elsevier Ireland Ltd. All rights reserved.

shown the internal or external wound repairing effects of *Hypericum perforatum* extracts. An experimental study on rats reported the increased healing of incision, excision and dead space wounds by oral administration of a *Hypericum perforatum* tincture (Rao et al., 1991). According to a previous clinical study, the oily extract of *Hypericum perforatum* combined with *Calendula arvensis* enhances healing of surgical wounds from cesarean section by augmentation in epithelial reconstruction (Lavagna et al., 2001). Recently, Süntar et al. (2010) have confirmed the significant in vivo wound-repairing activity of Turkish SJW olive oil extract (using dried and ground plants material) in excision and incision wound models. Two recent studies attempted to uncover the mechanism of cutaneous wound healing effect of SJW extracts. The aqueous alcohol extract of Turkish *Hypericum perforatum* extract was shown to stimulate the production of chicken embryonic fibroblast cells (Ozturk et al., 2007), whereas the crude methanol:acetone extracts of two *Hypericum perforatum* subspecies from Italy were found to increase the polygonal fibroblast migration and stimulation of collagen synthesis in NIH3T3 mouse fibroblast cells (Dikmen et al., 2011). However, to our knowledge, no study has attempted to investigate the detailed chemistry or the potential mechanism of action underlying the topical wound healing effect of the olive oil macerate from Turkish SJW at cellular and/or molecular levels.

In our recent study (Orhan et al., 2013), we performed qualitative and quantitative analyses on the secondary metabolites of olive oil macerates of SJW, i.e. naphthodiantrones (hypericin and pseudohypericin), acylated phloroglucinols (hyperforin and adhyperforin), flavonoids (quercetin and biapigenin) and chlorogenic acid by LC–DAD–MS. In the continuation of this project, we have now studied the fatty acid (FA) composition of 23 Turkish SJW oil macerates prepared either traditionally (home-made) or commercially (ready-made) by capillary GC–MS. Since all samples used in the present study were claimed to be prepared in olive oil, they were also investigated for chemical values (acidity, peroxide, iodine, saponification values, and the unsaponifiable matters) according to the methods established for olive oil by European Pharmacopoeia (2008). Furthermore, we hypothesized that the wound healing activity of these oil macerates could involve the inhibition of the key NF-κB cell signaling pathway, which is implicated in diverse cellular processes, including inflammation and wound healing (Mohanty et al., 2012). Hence, we studied the in vitro inhibitory effect of 10 home-made and 13 commercial SJW oil macerates against TNFα-induced NF-κB activation.

2. Materials and methods

2.1. Oil macerate samples of SJW

The purchase places and dates for home-made and commercial SJW oils are listed in Table 1.Samples **1**–**10** were obtained directly from the local people producing these oil macerates, whereas the rest of the samples were purchased from herbal shops.

2.2. Trans-methyl esterification of the oils

The SJW oil samples were separately weighed (450 mg) in 50-mL volumetric flasks, then saponified by adding 12 mL of 0.5N methanolic NaOH to the mixture and heated on a steam bath until the fat globules went into solution. 20 mL of BF_3/MeOH (Sigma Co.) were added into each flask and the mixtures were boiled for 2 min. After cooling down, they were made up to 50 mL with saturated NaCl solution, then were transferred to separatory funnels, and extracted with 30 mL of *n*-hexane for each. The *n*-hexane phases were taken and evaporated under vacuum. The obtained methyl esters of fatty acids were dissolved in 1 mL of *n*-hexane, 1 µL of which was used for GC–MS analysis.

2.3. GC–MS conditions for the oil macerate analyses

Chromatographic analysis of the oil macerates was carried out on Agilent 6890N Network GC system combined with Agilent 5973 Network Mass Selective Detector (GC–MS). The capillary column used was an Agilent 19091N-136 (HP Innowax Capillary; 60.0 m × 0.25 mm × 0.25 µm). Carrier gas was helium used as a flow rate of 0.8 mL/min with 1 µL injection volume. The samples were analyzed with the column held initially at 60 °C after injection during 10 min hold time, then temperature was increased to 220 °C with 4 °C/min heating ramp and kept at 220 °C for 10 min. Then final temperature was increased to 240 °C with 1 °C/min heating ramp. The injection was performed

Table 1
Date and place of purchases and producer companies (**11**–**23**) for SJW oil samples.

Oil no.	Dates purchased	Places purchased in Turkey	Producers of the SJW oils
1	November – 2006	Konya province	Home-made
2	April – 2006	Konya province	Home-made
3	April – 2007	Adana province	Home-made
4	April – 2007	Adana province	Home-made
5	April – 2007	Adana province	Home-made
6	April – 2007	Adana province	Home-made
7	August – 2006	Aydin province	Home-made
8	May – 2005	Manisa province	Home-made
9	May – 2005	Aydin province	Home-made
10	April – 2007	Ankara province	Home-made
11	April – 2007	Ankara province	Akhtar & Akhtar St. John's Wort oil
12	August – 2006	Aydin province	Bosphorus Flora St. John's Wort oil
13	November – 2006	Konya province	Kardelen St. John's Wort oil
14	November – 2006	Konya province	Karden St. John's Wort oil
15	September – 2006	Konya province	Talya St. John's Wort oil
16	September – 2006	Konya province	Sepe St. John's Wort oil
17	September – 2006	Konya province	Mecitefendi St. John's Wort oil
18	September – 2006	Konya province	Cemre St. John's Wort oil
19	April – 2007	Adana province	Defne/Doga St. John's Wort oil
20	April – 2007	Adana province	Nurs Lokman Hekim St. John's Wort oil
21	March – 2007	Hatay province	Sefer Yasemin St. John's Wort oil
22	March – 2007	Hatay province	Ege Lokman St. John's Wort oil
23	April – 2007	Aksaray province	Kirinti St. John's Wort oil

in split mode (50:1). Detector and injector temperatures were 230 °C and 280 °C, respectively. Run time was 80 min. MS scan range was (*m*/*z*): 35–450 amu (AMU) under electron impact (EI) ionization (70 eV). Identification of the peaks was achieved using Wiley and Nist Libraries, comparison of retention times (R_t) and of the mass spectra of standards (Sigma). Relative content of % fatty acids was determined with area under peaks using Agilent software. The results are expressed as an average of three determinations in all cases.

2.4. Determination of chemical values in the samples

Acid, peroxide, iodine, and saponification values as well as the unsaponifiable matter of the oil macerates were determined according to the methods described extensively in detail in European Pharmacopoeia (2008).

2.5. Cell culture and reagents

Human Philadelphia chromosome-positive chronic myelogenous leukemia cells (K562) were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (Hyclone, Perbio, Erembodegem, Belgium) and 1% (v/v) antibiotic–antimycotic (Lonza, BioWhittaker™, Verviers, Belgium) at 37 °C, in a 5% CO_2, humidified atmosphere. Human recombinant TNFα (PeproTech, Rocky Hill, NJ, USA) was resuspended in a phosphate buffer salt (PBS) 1 × sterile solution containing 0.5% bovine serum albumin (MP Biomedicals, Asse-Relegem, Belgium) to reach a final concentration of 10 μg/mL.

2.6. Transient transfections

Transient transfections of K562 cells were performed by electroporation using the BioRad gene Pulser according to published procedures (Duvoix et al., 2003). For each experiment, 3.75×10^6 cells at a concentration of 1.5×10^7 cells/mL were electroporated at the following settings: 250 V and 500 μF. For each transfection, 250 μL of cells were combined with 5 μg of firefly luciferase vector, NF-κB pGL4 (Promega, Leiden, The Netherlands), 5 μg of ph-RG-tk Renilla plasmid (Promega). 24 h after electroporation, transfected cells were harvested, re-suspended in growth medium to reach a final concentration of 10^6 cells/mL and may or may not have been subjected to the treatment by 0.02 μg/mL of TNFα for 6 h. In order to assay Luciferase and Renilla activity, 75 μL Dual-Glo™ Luciferase Reagent (Promega) were incubated for 10 min at 22 °C and then, 75 μL Dual-Glo™ Stop&Glo® Reagent (Promega) were added to the cells for 10 min incubation at 22 °C. Luciferase and Renilla activities were measured with an Orion microplate luminometer (Berthold detection systems) and results were expressed as a ratio of Luciferase activity normalized to Renilla activity.

3. Results

3.1. Fatty acid compositions in SJW oil macerates

The FA compositions of both the home-made (**1**–**10**) and the commercial type (**11**–**23**) oil macerates are displayed in Tables 2 and 3, respectively. As expected, the olive oil macerates of SJW were dominated by oleic acid (18:1), whose concentrations varied between 40.4 and 81.7% in home-made samples and 20.5 and 73.8% in commercial samples. In particular, traditionally-made sample **10** was outstanding with an oleic acid content of 81.7%. The linoleic acid (18:2) content was found to vary between 3.7 and 47.6% in home-made samples and 9.7 and 53.1% in commercial samples. Linolenic acid (18:3) was absent in all home-made oil~~s~~ samples, except for **6** (0.6%), but existed in low amounts in five oil macerates (**11**, **14**, **15**, **19**, and **22**) between 0.6 and 5.0%. The last unsaturated fatty acid (UFA) was palmitoleic acid (16:1) that was detected in four home-made oil macerates (**1**, **4**, **5**, and **6**) and five commercial samples (**13**, **16**, **18**, **21**, and **22**) at minute levels (0.4–0.9%). According to GC–MS data, the most predominant saturated FA (SFA) was palmitic acid (16:0) accounting between 8.3 and 14.7% of the home-made oils and 8.9 and 31.8% of the commercial oils. All samples were similar in their stearic acid (18:0) content, which was in the range of 2.1–3.7%. Interestingly, several oil macerates (e.g. **8**–**12** and **23**), lacked stearic acid. All home-made samples were devoid of myristic acid (14:0), and in fact, only six commercially obtained samples (**11**–**14**, **18**, and **19**) contained this FA at low levels (0.3–1.0%). Besides these highly variable individual FA levels, there were also great variations in the total saturated FA (SFA) and unsaturated FA (UFA) contents between home-made and commercial oil macerates. The SFA levels were between 11.6 and 17.9% in the former, and 12.1 and 32.8 in the latter. Similarly, the percentages of the UFA content varied between 83.4 and 88.4% in home-made samples, while the difference was much larger in the ready-made samples, ranging from 67.2 to 87.9%.

In order to achieve the quality control and to explore existence of any potential adulteration in these oil macerates, the values for acidity, peroxide, iodine, and saponification along with the unsaponifiable matters were calculated for all samples using the methods established by European Pharmacopoeia (2008) (Table 4). A great variation was observed among the mentioned values for each sample in comparison to those acceptable for olive oil by European Pharmacopoeia. As listed in Table 4, the values differed between 0.16 ± 0.01 and 4.87 ± 0.01 for acidity value (olive oil ≤ 2.0), 9.40 ± 0.08 and 155.86 ± 1.36 for peroxide value (olive oil ≤ 2.0), 73.89 ± 0.52 and 127.40 ± 1.22 for iodine value (olive oil 75–94), 110.40 ± 1.19 and

Table 2
Fatty acid composition of the traditionally-made SJW oil samples (mean values of relative % ± standard error). RT: Retention time of the fatty acid standards.

Oil no.	RT (minute)	1	2	3	4	5	6	7	8	9	10
Fatty acids											
Myristic (14:0)	18.56	–	–	–	–	–	–	–	–	–	–
Palmitic (16:0)	21.85	14.1 ± 0.08	14.3 ± 0.08	9.0 ± 0.00	12.9 ± 0.02	14.7 ± 0.27	10.9 ± 0.25	8.3 ± 0.04	13.6 ± 0.01	12.8 ± 0.00	14.6 ± 0.11
Stearic(18:0)	27.17	2.4 ± 0.15	2.1 ± 0.02	3.0 ± 0.01	3.5 ± 0.11	3.2 ± 0.16	2.4 ± 0.08	3.3 ± 0.04	–	–	–
Palmitoleic (16:1)	22.36	0.9 ± 0.29	–	–	0.7 ± 0.38	0.8 ± 0.09	0.8 ± 0.04	–	–	–	–
Oleic (18:1)	27.79	71.9 ± 0.45	72.9 ± 0.01	40.4 ± 0.02	68.8 ± 0.40	68.7 ± 0.25	75.5 ± 0.16	42.7 ± 0.01	72.1 ± 0.02	75.7 ± 0.04	81.7 ± 0.52
Linoleic(18:2)	29.37	10.6 ± 0.06	10.6 ± 0.01	47.6 ± 0.02	14.1 ± 0.12	12.7 ± 0.04	9.8 ± 0.02	45.7 ± 0.02	14.3 ± 0.02	11.4 ± 0.04	3.7 ± 0.42
Linolenic (18:3)	31.76	–	–	–	–	–	0.6 ± 0.00	–	–	–	–
Σ Saturated %	–	16.5	16.4	12.0	16.4	17.9	13.3	11.6	13.6	12.8	14.6
Σ Unsaturated %	–	83.4	83.5	88.0	83.6	86.1	86.7	88.4	86.4	87.1	85.4
Total (%)		99.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0	99.9	100.0

Table 3
Fatty acid composition of the commercial SJW oil samples (mean values of relative % ± standard error).

Oil no.	11	12	13	14	15	16	17	18	19	20	21	22	23
Fatty acids													
Myristic (14:0)	0.5 ± 0.01	1.0 ± 0.01	0.7 ± 0.01	0.3 ± 0.01	–	–	–	0.7 ± 0.01	0.5 ± 0.01	–	–	–	–
Palmitic(16:0)	23.0 ± 0.14	31.8 ± 0.06	26.7 ± 0.07	14.0 ± 0.03	8.9 ± 0.01	12.3 ± 0.01	13.7 ± 0.02	24.2 ± 0.28	22.7 ± 0.01	11.9 ± 0.01	10.8 ± 0.08	12.2 ± 0.09	14.3 ± 0.04
Stearic (18:0)	–	–	3.4 ± 0.02	2.4 ± 0.04	3.2 ± 0.02	2.9 ± 0.03	2.8 ± 0.02	2.3 ± 0.07	2.8 ± 0.01	2.1 ± 0.07	3.7 ± 0.09	2.6 ± 0.06	–
Palmitoleic (16:1)	–	–	0.5 ± 0.01	–	–	0.5 ± 0.01	–	0.5 ± 0.05	–	–	0.5 ± 0.04	0.4 ± 0.01	–
Oleic (18:1)	35.5 ± 0.35	51.2 ± 0.08	29.8 ± 0.01	58.2 ± 0.04	34.5 ± 0.01	55.0 ± 0.01	73.8 ± 0.06	20.5 ± 0.04	41.4 ± 0.01	32.9 ± 0.05	53.1 ± 0.00	73.2 ± 1.79	40.9 ± 0.04
Linoleic (18:2)	37.9 ± 0.25	16.0 ± 0.03	38.8 ± 0.09	20.0 ± 0.01	52.8 ± 0.01	29.3 ± 0.01	9.7 ± 0.02	51.6 ± 0.16	30.2 ± 0.01	53.1 ± 0.13	31.9 ± 0.06	11.0 ± 1.27	44.8 ± 0.08
Linolenic (18:3)	3.1 ± 0.04	–	–	5.0 ± 0.01	0.6 ± 0.01	–	–	–	2.4 ± 0.01	–	–	0.6 ± 0.03	–
Σ Saturated %	23.5	32.8	30.8	16.7	12.1	15.2	16.5	27.2	26.0	14.0	14.5	14.8	14.3
Σ Unsaturated %	76.5	67.2	69.1	83.2	87.9	84.8	83.5	72.6	74.0	86.0	85.5	85.2	85.7
Total (%)	100.0	100.0	99.9	99.9	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0

226.19 ± 1.80 for saponification value (olive oil 184–196), and 0.98 ± 0.02 and 8.14 ± 0.24 for unsaponifiable matter (olive oil ≤ 1.5). According to the results obtained, most of the oil macerates were shown to be out of the limits established for olive oil in European Pharmacopoeia (Table 4). Some extreme values were found in several of the oils such as oil **16** having peroxide value 155.86 ± 1.36, whose maximum limit should not be over 20.

3.2. TNFα-induced transcriptional activity of NF-κB by SJW oil macerates

The effect of oil macerates **1**–**23** on TNFα-induced transcriptional activity of NF-κB was examined using a luciferase reporter gene assay. Transiently transfected K562 cells were treated with the oil macerates (1000 μg/mL) for 2 h followed by a TNFα-treatment (0.02 μg/mL) stimulation during 6 h. Initial screening revealed that oils **1**, **3**–**5**, **7**, **9**-**12**, **14**, **17**–**22** had no effect on basal NF-κB transcription, whereas oil macerates **2**, **6, 8**, **13**, **15**, **16** and **23** reduced TNFα-induced NF-κB activation in a concentration-dependent manner. The active extracts were then further studied to obtain their NF-κB inhibitory (IC_{50}) values. The oil macerates showed moderate inhibitory activity, and the IC_{50} values (in μg/mL) were as follows: 175.7 ± 2.4 (**23**), 187.6 ± 21.3 (**6**), 204.6 ± 22.8 (**8**), 230.6 ± 51.8 (**15**), 239.2 ± 23.4 (**13**), 252.7 ± 16.5 (**16**), and 334.9 ± 34.4 (**2**) (Table 5). The control compound goniothalamin (Orlikova et al., 2013) was used at a concentration of 4 μg/mL, at which more than 90% of NF-κB activity was inhibited. Fig. 1 shows the activity of the individual samples **6** and **23** at different concentrations.

4. Discussion

This study dealt with quality control of SJW oil macerates sold in Turkey, presumably prepared in olive oil, by identification of fatty acid analysis and chemical values, plus explored potential mechanism of cutaneous wound healing activity. Three saturated FAs (myristic, palmitic, and stearic) and four unsaturated FAs (palmitoleic, oleic, linoleic, and linolenic) were identified in various levels. As discussed above, there were significant variations in the individual FA contents, as well as total saturated and unsaturated FA concentrations of the oils. As a note, there is only one publication that has determined the FA content of the petroleum ether extracts of several *Hypericum* species, including *Hypericum perforatum* (Stojanovic et al., 2003) that revealed palmitic acid to be the main SFA (20.3%), followed by lignoseric (15.3%) and stearic (8.7%) acids. The major UFA was oleic acid (17.1%), plus erucic (10.9%), linoleic (8.0%) and palmitoleic (3.3%) acids were detected. Here it needs to be mentioned that the FA contents of the oil macerates identified in this study are very likely to be due to oil (presumably olive oil) used for maceration. Since the amount of plant material used in the oil maceration is low, the FAs originating from the plant itself should be negligible.

As SJW is infused in olive oil, oleic acid, the most characteristic FA in olive oil, is expected to be the major FA of the SJW macerates. European Pharmacopoeia (2008) suggests olive oil to have the following composition:palmitic acid 7.5–20.0%, palmitoleic acid < 3.5%, stearic acid 0.5–5.0%, oleic acid 56.0–85.0%, and linoleic acid 3.5–20.0%. When we analyzed the FA compositions of 23 oil samples by GC–MS, we found out that oleic acid was the most abundant FA in majority of the home-made samples. Nevertheless, only two, out of ten, home-made oil macerates (**3** and **7**) failed to comply with the requirements of European Pharmacopoeia. Interestingly, both samples had comparable amounts of oleic and linoleic acids (ca. > 40%), suggesting that the oil used could be

Table 4
Acidity, peroxide, iodine and saponification values as well as the unsaponifiable matters in the SJW oil samples (mean values of relative % ± standard error).

Oil no.	Acid value	Peroxide value	Iodine value	Saponification value	Unsaponifiable matter
1	1.02 ± 0.06	56.95 ± 1.34	73.89 ± 0.52	217.12 ± 1.94	1.82 ± 0.09
2	1.00 ± 0.01	58.40 ± 0.85	74.40 ± 1.02	218.58 ± 0.71	1.81 ± 0.04
3	1.34 ± 0.04	36.90 ± 0.08	99.18 ± 1.16	207.51 ± 1.09	2.11 ± 0.13
4	2.81 ± 0.07	22.30 ± 0.45	77.50 ± 0.45	211.80 ± 1.78	1.86 ± 0.16
5	1.60 ± 0.07	38.17 ± 0.14	82.40 ± 0.85	200.72 ± 2.06	2.27 ± 0.03
6	1.64 ± 0.01	38.22 ± 1.39	111.44 ± 0.82	210.40 ± 0.42	2.07 ± 0.14
7	4.87 ± 0.01	20.42 ± 0.82	77.03 ± 0.83	216.40 ± 1.02	1.90 ± 0.02
8	1.70 ± 0.03	16.42 ± 0.30	78.90 ± 0.25	225.40 ± 0.66	2.39 ± 0.04
9	2.56 ± 0.01	26.02 ± 0.10	76.27 ± 0.49	226.19 ± 1.80	2.58 ± 0.15
10	1.60 ± 0.01	13.03 ± 0.34	78.74 ± 2.90	182.31 ± 1.33	8.14 ± 0.24
11	0.42 ± 0.01	15.82 ± 0.11	115.48 ± 0.59	182.20 ± 0.42	3.54 ± 0.23
12	2.82 ± 0.04	24.96 ± 0.11	87.91 ± 0.18	180.24 ± 2.16	2.13 ± 0.18
13	0.50 ± 0.03	9.71 ± 0.36	125.60 ± 0.57	183.96 ± 1.01	2.15 ± 0.09
14	0.56 ± 0.03	9.90 ± 0.23	127.40 ± 1.22	183.16 ± 0.59	2.25 ± 0.06
15	0.16 ± 0.01	18.16 ± 0.09	115.40 ± 0.88	207.79 ± 2.70	1.95 ± 0.06
16	4.63 ± 0.18	155.86 ± 1.36	95.72 ± 0.45	183.18 ± 1.29	1.39 ± 0.06
17	4.25 ± 0.07	23.10 ± 0.35	85.78 ± 0.17	193.30 ± 1.32	0.98 ± 0.02
18	0.50 ± 0.07	13.83 ± 0.61	100.61 ± 1.06	177.17 ± 2.01	1.67 ± 0.04
19	0.44 ± 0.04	19.22 ± 0.23	96.94 ± 0.25	180.60 ± 1.16	3.38 ± 0.14
20	1.50 ± 0.03	14.42 ± 0.20	100.40 ± 0.62	161.60 ± 3.00	2.44 ± 0.07
21	3.60 ± 0.07	9.40 ± 0.08	92.46 ± 0.96	176.30 ± 3.56	2.18 ± 0.19
22	1.44 ± 0.06	12.96 ± 0.42	78.42 ± 0.93	174.42 ± 2.40	2.35 ± 0.03
23	2.38 ± 0.11	27.08 ± 1.44	110.40 ± 1.19	110.40 ± 1.19	1.76 ± 0.07
Olive oil[a]	≤ 2.0	≤ 20	75–94	184–196	≤ 1.5

[a] Acceptable values for olive oil according to European Pharmacopoeia (2008).

Table 5
IC_{50} values for NF-κB inhibition potential of active oil macerates.

Oil no.	IC_{50} (μg/ml)
2	334.9 ± 34.4
6	187.6 ± 21.3
8	204.6 ± 22.8
13	239.2 ± 23.4
15	230.6 ± 51.8
16	252.7 ± 16.5
23	175.7 ± 2.4

another oil rather than olive oil (e.g. sunflower oil). The GC–MS results showed oleic acid percentage of the commercial oils generally to be lower than that required by European Pharmacopoeia for olive oil (Table 2). In fact, only three commercial samples (**14**, **17** and **22**) met the general requirements of European Pharmacopoeia for olive oil. The remaining ten samples had either almost 1:1 ratio of oleic:linoleic acids (samples **11**, **23**), or too high content of linoleic acid (**15**, **16**, **18**, **20**, **21**), or palmitic acid (**12**), or both linoleic and palmitic acids (**13**, **19**). This might be due to several reasons: i) either the traditional preparation has not been applied properly, or ii) a low quality olive oil was used, or iii) there is a possible adulteration of olive oil with other liquid oils since olive oil is quite expensive. Furthermore, the quality analyses of the oil macerates displayed in Table 4 shows that the values have significant deviations from those accepted for olive oil. These variations may again indicate the use of low quality liquid oil (s) for maceration, as well as the storage of the oil macerates in inappropriate conditions.

An additional important physicochemical property of Oleum Hyperici is the red color. A previous study stated that Oleum Hyperici does not contain hypericin, based on HPLC and TLC analyses, and the red color and fluorescence originate from lipophilic photo- and thermal degradation products of hypericin due to exposure to sunlight (Maisenbacher and Kovar, 1992). In a recent study, Isaachi et al. (2007) prepared a number of Oleum Hyperici samples by using different plant materials and techniques, and the LC–DAD–MS analyses resulted in the same conclusion. In our previous study (Orhan et al., 2013), we profiled the same oil samples used in the current study by LC–DAD–MS. Pseudohypericin and hypericin were detected in all samples, while hyperforin and adhyperforin were present, in variable amounts, only in the home-made samples. This might suggest a chemotypic variation, i.e. different chemical composition of the same plant growing in different geographical locations. In our study, all home-made samples had a dominant red color, whereas most of the commercial oils possessed various tones of yellow to pink colors. All these factors point at an adulteration and/or mispreparation in commercially sold Oleum Hyperici. Besides the adulteration of olive oil, it is drastically important to note that the samples may also suffer from adulteration or mis-identification of plant material, i.e. the use of another species of *Hypericum* rather than *Hypericum perforatum*. All these facts may underline the importance of using the traditional preparation method properly to obtain bioactive components as well as using the correct plant material.

Cutaneous wound healing is a complex physiological response of the epidermis to injury that relies on the coordinated action of diverse tissues, cells and mediators. Normally, it proceeds in an orderly way and overlapping events are characterized by three sequential phases, i.e. inflammatory phase, proliferation phase and tissue remodeling phase. Disruption of this cascade leads to delayed repair of acute wounds, prolonged inflammation, hence chronic wounds or ulcers (Ashcroft et al., 1999). The inflammation stage initiates after a wound is sealed by the formation of a blood clot, the so-called hemostasis (Midwood et al., 2004). Major cells of the immune system, in particular neutrophils, migrate and accumulate during the early inflammation of an acute wound area. Another characteristic of the inflammatory phase involves oxidative burst, i.e. the release of inflammatory mediators including reactive oxygen and nitrogen species (ROS and RNS, respectively) (Tsala et al., 2013). While ROS at high concentrations have pronounced bacteriostatic effects, at low concentrations they function as secondary messengers.

Tumor necrosis factor-alpha (TNFα), a pro-inflammatory cytokine, which is involved in regulation of inflammatory responses,

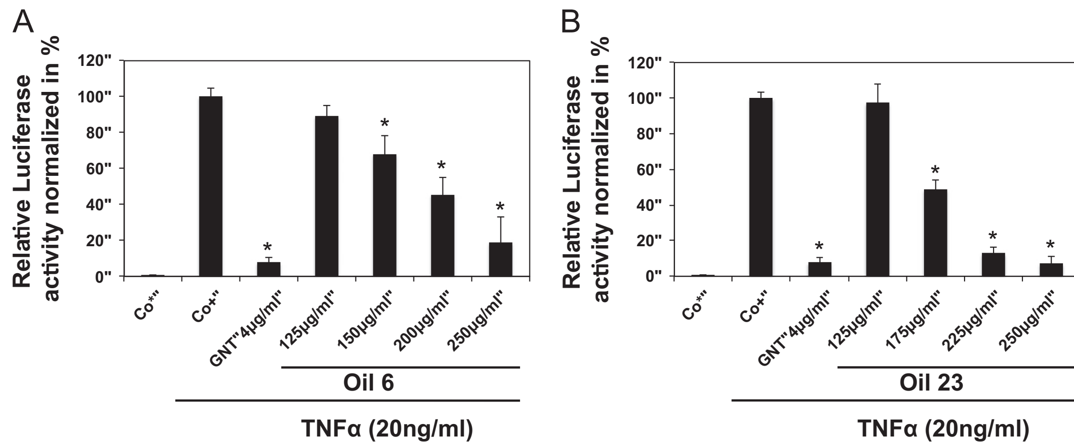

Fig. 1. Inhibition of TNFα-induced NF-κB activation by (**A**) SJW oil macerate **6**; (**B**) SJW oil macerate **23**. K562 cells are transiently transfected with firefly luciferase vector (NF-κB pGL4), and ph-RG-tk Renilla plasmid for 24 h. After transfection, K562 cells are treated with SJW oil macerates at the different concentrations for 2 h followed by a TNFα-treatment (0.02 μg/mL) during 6 h. Results are expressed as a ratio of the measured luminescence of the firefly luciferase vector and the luminescence of Renilla plasmid. Results are presented as a mean ± SD of three independent experiments. Negative control (Co^-) corresponds to DMSO treated cells, without TNFα activation while positive control (Co^+) corresponds to DMSO treated cells activated by TNFα. Goniothalamin (GNT) at indicated concentration was used as a positive inhibitory control. The asterisk indicates a significant difference compared to control positive as analyzed by *t*-test ($^*p < 0.05$).

cell cycle proliferation and apoptosis, is a key mediator during the inflammatory phase of wound healing (Han et al., 2001). TNFα levels significantly increase in both systemic and acute wounds (Ashcroft et al., 2012). The transcription factor NF-κB is a downstream factor of the TNF signaling pathway, playing a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation (TNFα, IL-1, VCAM-1, ICAM-1, iNOS). Upon an inflammatory stimulation or low levels of ROS, NF-κB translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, which leads to instant degradation of IκB-α, a group of proteins that limits NF-κB nuclear translocation (Perkins, 2007). The PI3K/AKT/NF-κB pathway is well known to contribute to inflammation during wound healing as the activation of NF-κB regulates inflammation and consequently delays the wound healing process (Mohanty et al., 2012). The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene and, therefore, independent luciferase and fluorescent translocation assays have been successfully used in drug discovery for several molecular targets (Nam, 2006). For all those reasons, NF-κB was selected as a potential molecular mechanism of the Turkish SJW oil samples. We show here for the first time that Turkish *Hypericum perforatum* oil macerates to inhibit TNFα-induced NF-κB activity in human leukemia cells. Although the IC_{50} values are moderate, it is still important that the inhibition of TNFα-induced NF-κB is likely involved in the wound healing effect of the oil samples.

Several studies have revealed TNFα-related effects of the individual constituents or the extracts of *Hypericum perforatum*. In an early study (Bork et al., 1999), hypericin was reported to inhibit phorbol ester- and TNFα-induced activation of NF-κB in HeLa and TC10 cells. Hyperforin, which was postulated to be the active metabolite of SJW (Maisenbacher and Kovar, 1992), was shown to induce IL-8 expression in human intestinal epithelia cells (IEC) and primary hepatocytes. This effect is MAPK-dependent, but independent from TNFα induced NF-κB pathway (Zhou et al., 2004). Tedeschi et al. (2003) reported that a commercial *Hypericum perforatum* extract shows anti-inflammatory properties by inhibiting cytokine-induced STAT-1α activation (but not NF-κB activation), thereby reducing iNOS expression. The authors also suggest that extracts from SJW may be a promising anti-inflammatory principle in chronic inflammatory diseases. In a mouse model of acute inflammation, Menegazzi et al. (2006) showed that inflammatory parameters (including lipid peroxidation, increased production of TNFα and IL-1β) were attenuated by *Hypericum perforatum* extract in carrageenan-induced lung injury. ICAM-1, nitrotyrosine, and poly (ADP-ribose), NF-κB and STAT-3 activation levels were significantly reduced in lung tissues. The same authors have recently demonstrated that *Hypericum perforatum* as well as hyperforin inhibit cytokine-elicited STAT-1 and NF-κB activation in isolated rat and human islets, hence point out the potential of SJW (and hyperforin) in the management of acute and chronical inflammation, e.g. chronical wounds in diabetes (Menegazzi et al., 2008). The available literature data indicates that the effect and probably mechanism of anti-inflammatory action of SJW and/or its constituents strongly depends on the cellular model used. In this study, Turkish *Hypericum perforatum* oil macerates inhibited TNFα-induced NF-κB activity in human leukemia cells clearly demonstrating new potential applications for both extracts and purified compounds related to hematological diseases.

On the other hand, olive oil or its major FA constituents have also been reported to show wound healing activity and/or deactivate NF-κB. In the study performed by Süntar et al. (2010), olive oil exerted some wound healing activity; however, the activity of the *Hypericum perforatum* oil preparation was significantly higher. Oral administration of oleic or linoleic acids accelerated the healing of inflammatory phase of wounds. Linoleic acid decreased the number of inflammatory cells and IL-1, IL-6 and macrophage inflammatory protein-3 concentrations, as well as NF-κB activation in the wound at 24 h post wounding (Rodrigues et al., 2012). Several groups have demonstrated that oleic acid modulates the inflammation by down-regulating COX2 expression and stimulating the production of cytokine-induced neutrophil chemoattractant (Cardoso et al., 2011). Furthermore, minor metabolites of the olive oil, e.g. secoiridoids and phenolics also inhibit NF-κB and exert anti-inflammatory activity (Sangiovanni et al., 2012). For instance; tyrosol, a typical phenolic compound in olive oil, was reported to decrease production of TNF-α and to inhibit LPS-induced NF-κB activation in lipopolysaccharide (LPS)-stimulated mice (Lu et al., 2013).

These data indicate that the topical wound healing effect of Oleum Hyperici to be very complex and likely to involve multiple mechanisms. The NF-κB inhibitory activity of the oil macerates is low, but this might be sufficient for the reduction of the inflammation particularly in the early stages of acute skin inflammation. The use of olive oil might be providing a synergistic and sustainable NF-κB inhibition (and/or other mechanisms) and topical

wound healing effects. Furthermore, the well-known antibacterial and antioxidant effects of both olive oil and SJW might be also involved in the repair of the wound.

5. Conclusions

Wound-healing activity of the olive oil extract of *Hypericum* species is known since ancient times, but in comparison to antidepressant and other CNS activities of the plant, only a few studies have been carried out on this type of effect. In a previous study, we analyzed the content of several bioactive compound classes, naphthodiantrones, acylated phloroglucinols, flavonoids and chlorogenic acid in SJW oil macerates by LC–DAD–MS. Herein, we have identified the FA composition and performed quality control of the same SJW oils, plus explored the potential wound healing mechanism of the oils. We have previously shown that Turkish SJW oils contain bioactive constituents (e.g. hypericin, pseudohypericin, hyperforin, adhyperforin, and flavonoids). These metabolites plus the FAs found in olive oil may be responsible for the anti-inflammatory activity of the Oleum Hyperici samples. Our results underline that the traditional way of preparing infused oil from *Hypericum perforatum* using olive oil is capable of getting bioactive constituents in the oil if prepared, stored and applied properly.

Acknowledgments

A part of this study is the master thesis of Gulin Yetkin (B. Pharm.) entitled as "Phytotherapeutic Researches on St. John's Wort Oils Commercially Sold in Turkey" which was completed at the Institute of Health Sciences of Gazi University (Ankara, Turkey). Research at the Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC) is financially supported by "Recherche Cancer et Sang" foundation, by Recherches Scientifiques Luxembourg asbl , by Een Häerz fir Kriibskrank Kanner association and the Action Lions "Vaincre le Cancer"Luxembourg, MD and BO are supported by the National Research Foundation (NRF) by the MEST of Korea for Tumor Microenvironment Global Core Research Center (GCRC) Grant number 2012-0001184; by the Seoul National University Research grant and by the Research Settlement Fundfor the new faculty of SNU.

References

Ashcroft, G.S., Greenwell-Wild, T., Horan, M.A., Wahl, S.M., Ferguson, M.W., 1999. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. American Journal of Pathology 155, 1137–1146.

Ashcroft, G.S, Jeong, M.J., Ashworth, J.J., Hardman, M., Jin, W., Moutsopoulos, N., Wild, T., McCartney-Francis, N., Sim, D., McGrady, G., Song, X.Y., Wahl, S.M., 2012. Tumor necrosis factor-alpha (TNF-α) is a therapeutic target for impaired cutaneous wound healing. Wound Repair and Regeneration 20, 38–49.

Baytop, T., 1984. Therapy with Herbs in Turkey. Istanbul Univ. Press, Istanbul, pp. 185–186 (Publication no. 3255).

Bork, P.M., Bacher, S., Schmitz, M.L., Kaspers, U., Heinrich, M., 1999. Hypericin as a non-antioxidant inhibitor of NF-κB. Planta Medica 65, 297–300.

Cardoso, C.R., Favoreto Jr., S., Oliveira, L.L., Vancim, J.O., Barban, G.B., Ferraz, D.B., Silva, J.S., 2011. Oleic acid modulation of the immune response in wound healing: a new approach for skin repair. Immunobiology 216, 409–415.

Dikmen, M., Ozturk, Y., Sagratini, G., Ricciutelli, M., Vittori, S., Maggi, F., 2011. Evaluation of the wound healing potentials of two subspecies of *Hypericum perforatum* on cultured NIH3T3 fibroblasts. Phytotherapy Research 25, 208–214.

Duvoix, A., Morceau, F., Delhalle, S., Schmitz, M., Schnekenburger, M., Galteau, MM., Dicato, M., Diederich, M., 2003. Induction of apoptosis by curcumin: mediation by glutathione S-transferase P1-1 inhibition. Biochemical Pharmacology 66, 1475–1483.

European Pharmacopeia. 2008. Council of Europe, 4th Edition, Strasbourg.

Francis, A.J. P., 2005. Antidepressant action of St. John's wort, *Hypericum perforatum*: a test of the circadian hypotheses. Phytomedicine 12, 167–172.

Franco, M.N., Galeano-Diaz, T., Sanchez, J., De Miguel, C., Martin-Ventedor, D., 2014. Total phenolic compounds and tocopherols profiles of seven olive oil varieties grown in the south-west of Spain. J. Oleo Science 63, 115–125.

Han, Y.P., Tuan, T.L., Wu, H., Hughes, M., Garner, W.L., 2001. TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. Journal of Cell Science 114, 131–139.

Isaachi, B., Bergonzi, M.C., Carnevali, F., van der Esch, S.A., Vincieri, F.F., Bilia, A.R, 2007. Analysis and stability of the constituents of St. John's Wort oils prepared with different methods. J. Pharmaceutical and Biomedical Analysis 45, 756–761.

Lavagna, S.M., Secci, D., Chimenti, B., Bonsignore, L., Ottaviani, A., Bizzarri, B., 2001. Efficacy of *Hypericum* and *Calendula* oils in the epithelial reconstruction of surgical wounds in childbirth with caesarean section. Il Farmaco 56, 451–453.

Lu, J., Huang, G., Wang, Z., Zhuang, Z., Xu, L., Song, B., Xiong, B., Guan, S., 2013. Tyrosol exhibits negative regulatory effects on LPS response and endotoxemia. Food Chemical Toxicology 62, 172–178.

Maisenbacher, P., Kovar, K.A., 1992. Analysis and stability of *Hyperici oleum*. Planta Medica 58, 351–354.

Menegazzi, M., Di Paola, R., Mazzon, E., Muià, C., Genovese, T., Crisafulli, C., Suzuki, H., Cuzzocrea, S., 2006. *Hypericum perforatum* attenuates the development of carrageenan-induced lung injury in mice. Free Radical Biology and Medicine 40, 740–753.

Menegazzi, M., Novelli, M., Beffy, P., D'Aleo, V., Tedeschi, E., Lupi, R., Zoratti, E., Marchetti, P., Suzuki, H., Masiello, P., 2008. Protective effects of St. John's wort extract and its component hyperforin against cytokine-induced cytotoxicity in a pancreatic beta-cell line. International Journal of Biochemistry Cell Biology 40, 1509–1521.

Midwood, K.S., Williams, L.V., Schwarzbauer, J.E., 2004. Tissue repair and the dynamics of the extracellular matrix. International Journal of Biochemistry Cell Biology 36, 1031–1037.

Mohanty, C., Das, M., Sahoo, S.K., 2012. Sustained wound healing activity of curcumin loaded oleic acid based polymeric bandage in a rat model. Molecular Pharmaceutics 9, 2801–2811.

Nam, N.H., 2006. Naturally occurring NF-kappaB inhibitors. Mini Reviews in Medicinal Chemistry 6, 945–951.

Orhan, I.E., Kartal, M., Gülpinar, A.R., Cos, P., Matheeussen, A., Maes, L., Tasdemir, D., 2013. Assessment of antimicrobial and antiprotozoal activity of the olive oil macerate samples of *Hypericum perforatum* and their LC-DAD-MS analyses. Food Chemistry 138, 870–875.

Orlikova, B., Schumacher, M., Juncker, T., Yan, C.C., Inayat-Hussain, S.H., Hajjouli, S., Cerella, C., Dicato, M., Diederich, M., 2013. Styryl-lactone goniothalamin inhibits TNF-α-induced NF-κB activation. Food and Chemical Toxicology 59, 572–578.

Ozturk, N., Korkmaz, S., Ozturk, Y., 2007. Wound-healing activity of St. John's Wort (*Hypericum perforatum* L.) on chicken embryonic fibroblasts. Journal of Ethnopharmacology 111, 33–39.

Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-κB and IKK function. Nature Reviews 8, 49–62.

Rao, S.G., Udupa, A.L., Udupa, S.L., Rao, P.G.M., Rao, G., Kulkarni, D.R., 1991. *Calendula* and *Hypericum:* two homeopathic drugs promoting wound healing in rats. Fitoterapia 6, 508–510.

Rodrigues, H.G., Vinolo, M.A., Magdalon, J., Vitzel, K., Nachbar, R.T., Pessoa, A.F., dos Santos, M.F., Hatanaka, E., Calder, P.C., Curi, R., 2012. Oral administration of oleic or linoleic acid accelerates the inflammatory phase of wound healing. Journal of Investigative Dermatology 132, 208–215.

Sangiovanni, E., Colombo, E., Fumagalli, M., Abbiati, F., Caruso, D., Dell'Agli, M., 2012. Inhibition of NF-κB activity by minor polar components of extra-virgin olive oil at gastric level. Phytotherapy Research 26, 1569–1571.

Stojanovic, G., Palic, R., Tarr, C.H., Reddy, C.M., Marinkovic, C., 2003. *n*-Alkanes and fatty acids of *Hypericum perforatum*, *Hypericum maculatum*, *Hypericum olympicum*. Biochemical Systematics and Ecology 31, 223–226.

Süntar, I.P., Akkol, E.K., Yilmazer, D., Baykal, T., Kirmizibekmez, H., Alper, M., Yesilada, E., 2010. Investigations on the in vivo wound healing potential of *Hypericum perforatum L.* Journal of Ethnopharmacology 127, 468–477.

Tedeschi, E., Menegazzi, M., Margotto, D., Suzuki, H., Forstermann, U., Kleinert, H., 2003. Anti-inflammatory actions of St. John's wort: inhibition of human inducible nitric-oxide synthase expression by down-regulating signal transducer and activator of transcription-1alpha (STAT-1alpha) activation. Journal of Pharmacology and Experimental Therapeutics 307, 254–261.

Tsala, D.E., Amadou, D., Habtemariam, S, 2013. Natural wound healing and bioactive natural products. Phytopharmacology 4, 532–560.

Yesilada, E., Honda, G., Sezik, E., Tabata, M., Fujita, T., Tanaka, T., Takeda, Y., Takaishi, Y., 1995. Traditional medicine in Turkey. V. Folk medicine in the inner Taurus Mountains. Journal of Ethnopharmacology 46, 133–152.

Zhou, C., Tabb, M.M., Sadatrafiei, A., Grun, F., Sun, A., Blumberg., B., 2004. Hyperforin, the active component of St. John's wort, induces IL-8 expression in human intestinal epithelial cells *via* a MAPK-dependent, NF-kappaB-independent pathway. Journal of Clinical Immunology 24, 623–636.